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Evidence Supporting the Role of DNA Pyridyloxobutylation in Rat Nasal Carcinogenesis by Tobacco-specific Nitrosamines[1]

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ABSTRACT

The tobacco-specific nitrosamines 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N'-nitrosonornicotine (NNN) both induce nasal tumors in rats and have a common metabolic activation pathway leading to pyridyloxobutylation of DNA. The role of DNA pyridyloxobutylation in rat nasal carcinogenesis has not been evaluated previously. In this study, we used gas chromatography-mass spectrometry to compare levels of 4-hydroxy-1-(3-pyridyl)-1-butanone-releasing adducts formed by pyridyloxobutylation of rat nasal mucosa DNA after treatment with either NNK, NNN, or deuterated analogues of NNK. The latter were [4,4-D_2]NNK, a stronger nasal cavity carcinogen than NNK, and [CD_3]NNK, which has carcinogenic activity equivalent to NNK. We also investigated toxicity to the nasal mucosa and levels of O^6-methylguanine in the DNA of this tissue in rats treated with NNK and its deuterated analogues. Rats were given three times weekly s.c. injections of the respective nitrosamines for 4 weeks and then sacrificed 24 h after the final injection. The nasal mucosa was separated into the olfactory and respiratory portions. In the rats treated with [4,4-D_2]NNK, levels of O^6-methylguanine in DNA from both the olfactory and respiratory portions of the nasal mucosa were significantly lower and levels of 4-hydroxy-1-(3-pyridyl)-1-butanone-releasing DNA adducts higher than in the rats treated with equivalent doses of the less carcinogenic compounds NNK or [CD_3]NNK. 4-Hydroxy-1-(3-pyridyl)-1-butanone-releasing adducts were also detected in the nasal mucosa DNA of the rats treated with NNN. In the comparative study of NNK and its deuterated analogues, the histology of the nasal mucosa did not appear to be markedly different among these groups. Collectively, the results of this study provide strong evidence that DNA pyridyloxobutylation is important in rat nasal cavity carcinogenesis by NNK and NNN.

INTRODUCTION

NNK[3] and NNN are tobacco-specific nitrosamines that have been extensively studied because of their likely involvement in human cancers induced by tobacco products (1–3). The lung is the major site of tumor production in rodents treated with NNK. Esophageal tumors are produced when NNN is administered to rats in the drinking water. While lung tumors are seldom induced in rats by NNN and esophageal tumors are virtually unknown in rats treated with NNK, these two nitrosamines do have one target tissue in common, the nasal mucosa of rats. Both compounds induce benign and malignant tumors of the nasal mucosa when administered by s.c. injection at total doses of 3 mmol/kg or greater. At total doses of 1 mmol/kg, both compounds induce predominantly benign tumors of the nasal mucosa (Table 1; Refs. 4–6). Lower doses of NNK produce mainly lung tumors (5, 7). The malignant tumors induced in the rat nose by NNK and NNN arise mainly from the olfactory portion of the nasal mucosa (8, 9). The potencies of NNK and NNN for induction of nasal tumors in rats are comparable (Table 1). The induction of nasal tumors by NNK and NNN is an important end point for understanding the mechanisms of carcinogenesis by these nitrosamines because the rat nasal mucosa is rich in enzymes which metabolically activate NNK and NNN.

Major known metabolic activation pathways of NNK and NNN are summarized in Fig. 1 (1, 2). Hydroxylation of NNK at the α-methylene group leads to 4-oxo-1-(3-pyridyl)-1-butanone and methanediazohydroxide. The latter reacts with DNA producing a variety of methyl adducts, among which are O^6-mG and 7-methylguanine. O^6-mG is known to have miscoding properties and is hypothesized to be important in carcinogenesis by NNK (9–12). Hydroxylation of the α-methyl group of NNK produces formaldehyde and 4-(3-pyridyl)-4-oxobutanediazohydroxide. This diazohydroxide reacts with H_2O to form HPB and with DNA to produce pyridyloxobutyl DNA adducts (13, 14). The major adducts formed in this reaction can be released from the DNA as HPB by acid hydrolysis. α-Hydroxylation of NNN at its 2′ carbon also yields 4-(3-pyridyl)-4-oxobutanediazohydroxide and DNA adducts that release HPB upon acid hydrolysis, as in the case of NNK. α-Hydroxylation of NNN at its 5′ carbon is a known metabolic pathway, but DNA adducts resulting from this pathway have not been detected. In a previous study using cultured rat nasal mucosa, we showed that NNK and NNN were extensively metabolized by α-hydroxylation (15). The results of that study indicated that both α-hydroxylation pathways of both nitrosamines were catalyzed by enzymes in the rat nasal mucosa. Subsequent investigations using rat and rabbit nasal microsomes have demonstrated that α-hydroxylation of NNK is mediated by cytochrome P-450 enzymes (16–18).

The respective roles in rat nasal carcinogenesis by NNK of the pathways leading to DNA methylation and pyridyloxobutylation have not been clearly defined. Belinsky *et al.* (9) concluded that formation of O^6-mG, together with toxicity in the olfactory portion of the nose, was important in the expression of rat nasal carcinogenesis by NNK. It seemed logical to us that DNA pyridyloxobutylation would be involved in tumor induction by NNK and NNN since they have similar carcinogenic properties toward the rat nasal mucosa and have a common metabolic pathway leading to DNA pyridyloxobutylation. Several lines of evidence support this hypothesis: (*a*) as indicated above, NNK and NNN are both extensively metabolized by this pathway in rat nasal tissues (15); (*b*) NNK and NNN are more carcinogenic to the rat nasal mucosa than is NDMA, which only methylates DNA but does not pyridyloxobutylate (see Table 1). In contrast, NNN does not methylate DNA (19); (*c*) model compounds such as CNPB and NNKOAc which can only pyridyloxobutylate DNA have been shown to be highly mutagenic in both bacterial and mammalian systems indicating that the DNA adducts formed by this pathway, although structurally uncharacterized, are biologically important (20, 21); and (*d*) in rat liver and lung, pyridyloxobutylated DNA has been shown to persist for up to 4 weeks (22).

Since pyridyloxobutylation of rat nasal mucosa DNA had not been previously demonstrated *in vivo*, we used a newly developed GC-MS assay in this study to investigate its potential role in rat nasal carcinogenesis by NNK and NNN (23). This assay is highly sensitive,

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[3] The abbreviations used are: NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N'*-nitrosonornicotine; NDMA, *N*-nitrosodimethylamine; O^6-mG, O^6-methylguanine; HPB, 4-hydroxy-1-(3-pyridyl)-1-butanone; CNPB, 4-(carbethoxymethylnitrosamino)-1-(3-pyridyl)-1-butanone; NNKOAc, 4-(acetoxymethylnitrosamino)-1-(3-pyridyl)-1-butanone; GC-MS, combined gas chromatography-mass spectrometry; HPP, 5-hydroxy-1-(3-pyridyl)-1-pentanone; NMR, nuclear magnetic resonance.

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Table 1 *Nasal cavity carcinogenesis in male F344 rats by tobacco-specific nitrosamines and related compounds*[a]

Compound	Single dose (mg/kg)	Total dose (mmol/kg)	Effective # of rats	No. of rats with nasal cavity tumors (%)			Reference
				Benign	Malignant	Total	
NNK	1.14	0.33	27	5 (19)	1 (4)	6[b] (22)	5
	3.45	1.0	28	11 (39)	2 (7)	13 (46)	6
	10.35	3.0	15	6 (40)	7 (47)[c]	13 (87)[c]	6
NDMA	0.41	0.33	27	1 (4)	0 (0)	1 (4)	5
NNN	2.95	1.0	27	11 (41)	4 (15)	15 (56)	4
	8.85	3.0	15	3 (20)	8 (53)[d]	11 (73)	4
[4,4-D_2]NNK	1.15	0.33	23	8 (35)	4 (17)	12 (52)[e]	6
	3.48	1.0	26	12 (46)	9 (35)[f]	21 (81)[g,h]	6
[CD_3]NNK	1.16	0.33	29	2 (7)	2 (7)	4 (14)	6
	3.50	1.0	30	15 (50)	6 (20)	21 (70)	6

[a] Male F344 rats were treated 3 times weekly for 20 weeks with NNK by s.c. injection in trioctanoin. Bioassays were terminated after 2 years.
[b] Significantly greater than NDMA, $P < 0.05$.
[c] Significantly greater than NNK at 1.0 and 0.33 mmol/kg, $P < 0.05$.
[d] Significantly greater than NNN at 1.0 mmol/kg, $P < 0.01$.
[e] Significantly greater than NNK at 0.33 mmol/kg, $P < 0.05$.
[f] Significantly greater than NNK at 1.0 mmol/kg, $P < 0.05$.
[g] Significantly greater than NNK at 1.0 mmol/kg, $P < 0.01$.
[h] Mean survival significantly less than NNK or [CD_3]NNK at 1.0 mmol/kg, $P < 0.05$.

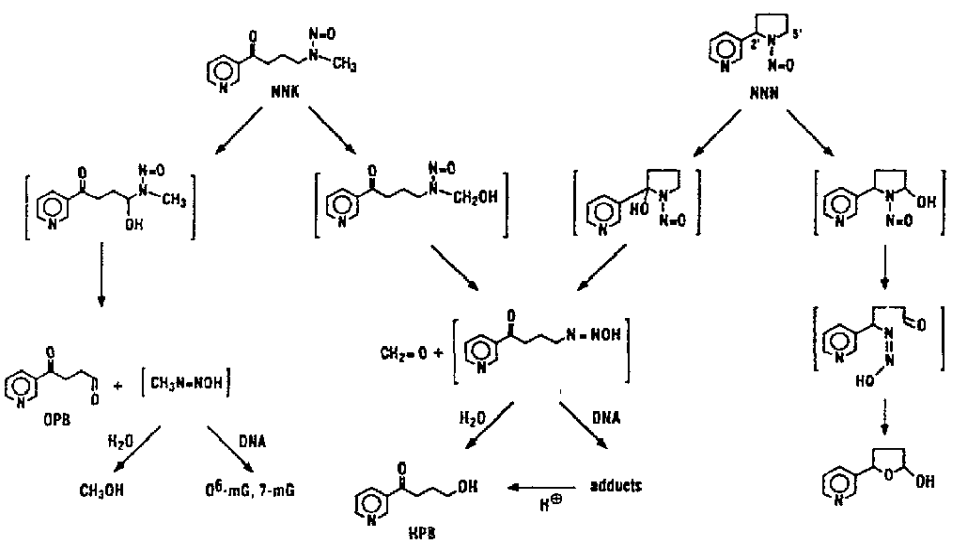

Fig. 1. Major known metabolic activation pathways of NNK and NNN.

allowing quantitation of HPB released from relatively small samples of DNA, such as those obtained from rat nasal mucosa. We have compared levels of DNA pyridyloxobutylation in the respiratory and olfactory portions of the rat nasal mucosa after 4 weeks of treatment with either NNK, NNN, or two deuterated analogues of NNK, [4,4-D_2]NNK and [CD_3]NNK (Fig. 2). Previously, we had shown that [4,4-D_2]NNK was more carcinogenic than either NNK or [CD_3]NNK toward the rat nasal mucosa (Table 1; Ref. 6). In the rats treated with [4,4-D_2]NNK, we would have expected lower levels of O^6-mG than in rats treated with NNK or [CD_3]NNK due to inhibition of α-methylene hydroxylation as a consequence of the deuterium isotope effect. As this did not seem to agree with the proposal by Belinsky *et al.* (9) that O^6-mG was important in rat nasal carcinogenesis by NNK, we have therefore also compared levels of O^6-mG in the olfactory and respiratory portions of the rat nasal mucosa upon treatment with NNK and its deuterated analogues.

MATERIALS AND METHODS

Chemicals. NNK, NNN, [4,4-D_2]NNK, and [CD_3]NNK were synthesized as described (6, 24). ^{1}H-NMR established that the isotopic purities of the deuterated compounds were greater than 99%. HPP was prepared from δ-valerolactone (Aldrich Chemical Co., Milwaukee, WI) and ethyl nicotinate (Aldrich) by a method similar to that used for HPB (25). It was purified by silica gel chromatography and obtained as an oil. It was characterized by its spectral properties: ^{1}H-NMR δ ($CDCl_3$), 1.6–1.7 (2H, m, CH_2); 1.78–1.88 (2H, m CH_2); 3.03 (2H, t, J = 7.15 Hz, CH_2CO); 3.66 (2H, t, J = 6.33 Hz, CH_2OH). 7.4 (1H, dd, Pyr-H_5, $J_{4,5}$=$J_{5,6}$=7.83 Hz); 8.2 (1H, d, Pyr-H_4, $J_{4,5}$=7.83Hz); 8.72 (1H, bs, Pyr-H_6); 9.1 (1H, bs, Pyr-H_2); and MS, m/z (rel intensity), 179 (M^+,0.6); 162 (80); 106 (100); and 78 (36). [CD_3]O^6-mG was prepared from CD_3OD (Aldrich) and 6-chloroguanine as described for O^6-mG (26). It was characterized by its ^{1}H-NMR δ (DMSO-d_6), 6.30 (2H, s, NH_2); 7.85 (1H, s, H_8); 12.42 (1H, s, H_9); and MS (desorption chemical ionization mode) m/z (rel intensity), 169 (M+1, 100); 115 (44); 82 (60); and 71 (62).

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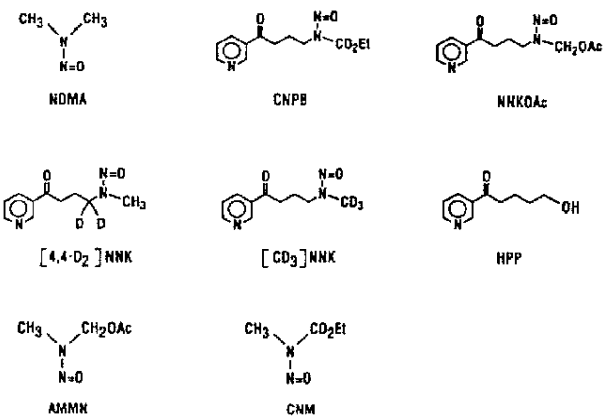

Fig. 2. Structures of compounds discussed in the text.

Animal Treatments. Male F344 rats were obtained from Charles River Breeding Laboratories, Kingston, NY. They were housed under standard conditions as described (4). In experiment 1, four rats per group were each given s.c. injections of 0.0167 mmol/kg NNK, NNN, [4,4-D_2]NNK, or [CD_3]NNK in 0.5 ml of trioctanoin or 0.5 ml of trioctanoin alone. Injections were carried out 3 times weekly for 4 weeks. Twenty-four h after the final injection, the rats were sacrificed, and the olfactory and respiratory portions of the nasal mucosa were excised for DNA isolation and analysis of pyridyloxobutylation. An identical protocol was used for experiment 2, except that each group consisted of eight rats. DNA was isolated from the respiratory and olfactory portions of the nasal mucosa of four rats and analyzed for O^6-mG. The nasal passages of the other four rats were evaluated for histological changes. An additional group of 12 rats was treated with 0.048 mmol/kg NNK and evaluated for toxicity toward the nasal mucosa as well as pyridyloxobutyl and O^6-mG adducts.

Isolation of Respiratory and Olfactory Nasal Mucosa. Rats were anesthetized with CO_2 and sacrificed by decapitation. The mandibula was removed, and the head was placed on the necropsy board with the palate facing up. The head was split using a bone mallet and a scalpel, cutting longitudinally on the median line, through the hard palate. Respiratory mucosa was retrieved from the naso- and maxilloturbinates, the lateral walls of the nasal passages, and the median septum anterior to the olfactory area. Olfactory mucosa was obtained from the ethmoturbinates and the lateral wall and septum of the olfactory area. One rat was used for each DNA isolation from respiratory and olfactory mucosa.

DNA Isolation. DNA was isolated from the respiratory and olfactory mucosa using a modification of the method described by Murphy (27). The tissue was minced and homogenized by hand in 1.5 ml of 0.15 M NaCl-0.015 M sodium citrate buffer, pH 7.0. The homogenizer was rinsed with three 1-ml portions of buffer, and the washings were combined with the homogenate. Fifty μl of 10% sodium dodecyl sulfate were added to this solution. After 15 min at room temperature, the solution was incubated for 1 h at room temperature with 0.1 mg RNase Type III A from bovine pancreas (Sigma Chemical Co., St. Louis, MO) and 50 units of RNase T1 from *Aspergillus oryzae*, followed by incubation with 1 mg of proteinase K, Type XI, for 1 h. The resulting mixture was extracted with 5:1 chloroform:isoamyl alcohol for 15 min and then subjected to centrifugation at 8500 × *g* for 15 min at 4°C. The aqueous layer was removed and concentrated under vacuum to a volume of less than 2 ml. This solution was then extracted again with chloroform:isoamyl alcohol. The aqueous portion was removed and treated with 50 μg RNase Type IIIA and 25 units of RNase T1 for at least 1 h. The DNA was then isolated on a Protein Pak 5PW high-performance liquid chromatography column and desalted on a Bio Gel P6DG desalting column as described (27). In the preparation of the DNA from experiment 2, we noticed that not all of the DNA eluted from the column under normal gradient conditions. The remaining DNA did elute upon injection of 0.1 ml of 0.2 N NaOH, following elution of the main DNA peak. The identity of this material as DNA was confirmed by hydrolysis to nucleosides in control experiments with calf thymus DNA. In some of the nasal mucosa DNA samples, both DNA fractions were analyzed for O^6-mG, and the results were combined. Control experiments demonstrated that O^6-mG was stable in DNA under the conditions of base elution from the column.

Analysis of DNA for O^6-mG. DNA was hydrolyzed by heating in 0.1 N HCl at 80°C for 1 h. After cooling to room temperature, the samples were analyzed for O^6-mG and guanine, essentially as described (28). The high-performance liquid chromatography system consisted of a Perkin Elmer Model 250 binary LC Pump, a Perkin Elmer LC 240 fluorescence detector, a Rheodyne Model 7125 injector and a 4.6 mm x 250 mm Phenomenex Partisil 10 SCX column. For analysis of DNA from the rats treated with [CD_3]NNK, [CD_3]O^6-mG was synthesized as described above and used for quantitation. Differences among the groups were analyzed statistically by a log transformed *t* test.

Analysis of DNA for HPB-releasing Adducts. DNA was hydrolyzed in 0.1 N HCl at 80°C for 3 h. The protons at the 4-position of HPB do not exchange under these conditions, as determined by NMR. The samples were allowed to cool to room temperature and a portion was removed for guanine analysis. The remainder was analyzed for HPB released essentially as described (23). Analysis of the DNA isolated from the animals treated with [4,4-D_2]NNK required the use of an internal standard other than that which is normally used, [4,4-D_2]HPB. For this purpose, we used HPP. It was added to all samples from the group treated with [4,4-D_2]NNK, and its presence was monitored by selected ion monitoring at m/z 373. Control experiments demonstrated that HPP behaved in the assay essentially the same as HPB.

A sample was determined to have a detectable level of HPB if the ratio of the signal at m/z 359, or m/z 361 in the case of [4,4-D_2]NNK, was at least twice as great as the corresponding signal in either the blank or control DNA sample run with that particular set. Differences among the groups were analyzed by a Newman-Keuls multiple comparisons test.

Histology of the Nasal Mucosa. The nasal cavities of four animals in each group were examined microscopically for morphological changes that could be attributed to specific NNK toxicity. After removal of the mandibula, the head was fixed in buffered formalin and decalcified. Four equidistant frontal sections were made through the nasal cavity, and the parts obtained were processed for histology after paraffin embedding. Hematoxylin and eosin and periodic acid Schiff staining were performed.

RESULTS

In the carcinogenicity studies carried out previously, rats were treated three times weekly for 20 weeks with s.c. injections of NNN, NNK, or its deuterated analogues to give total doses as illustrated in Table 1 (6). In the present study, we used the same s.c. injection protocol for 4 weeks since previous results indicated that steady state levels of DNA adducts would be obtained in that time (7, 9, 29). Thus, the results should be indicative of adduct levels during the chronic treatment protocol used in the carcinogenicity assays.

Levels of O^6-mG and HPB released from the olfactory and respiratory mucosa DNA of rats treated with NNK, its deuterated analogues, and NNN are summarized in Table 2. The highest levels of O^6-mG were found in the DNA of the olfactory mucosa of the rats to which NNK and [CD_3]NNK were administered at 3.46–3.51 mg/kg. These levels were 4–6 times higher than in the corresponding respiratory mucosa DNA and 16–26 times higher than in the olfactory mucosa DNA of the rats treated with an equimolar dose of [4,4-D_2]-NNK. The levels of O^6-mG in the olfactory mucosa DNA of the rats given the lower dose of NNK were also greater than those in the olfactory mucosa DNA of the rats treated with the higher dose of NNK. In the respiratory mucosa DNA, levels of O^6-mG were approximately four times higher in the rats treated with NNK and [CD_3]NNK than in the rats given [4,4-D_2]NNK.

HPB-releasing adduct levels were near the detection limit in the olfactory and respiratory mucosa DNA of the rats treated with the lower dose of NNK and with [CD_3]NNK. In contrast, HPB-releasing adducts were readily detectable in all rats treated with [4,4-D_2]NNK (Fig. 3). The levels of HPB-releasing adducts were thus clearly higher

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Table 2 *Levels of O^6-mG and HPB-releasing adducts in nasal mucosa DNA of F344 rats treated with tobacco-specific nitrosamines and analogues*[a]

Group	Single dose mg/kg[c]	pmol/μmol guanine[b] O^6-mG		HPB released		HPB:O^6-mG	
		Olfactory	Respiratory	Olfactory	Respiratory	Olfactory	Respiratory
NNK	3.46	235 ± 161 (n = 4)	54.3 ± 33.6 (n = 4)	<0.4[d], <0.8, 2.5	<0.4, <2.0, <1.6, 4.5	<0.005	<0.04
NNK	9.94	19.8 ± 3.4[e] (n = 3)	29.9 ± 12.2 (n = 3)	4.0 ± 1.6 (n = 4)	3.9 ± 2.6 (n = 4)	0.20	0.13
[4,4-D_2]NNK	3.49	14.6 ± 3.7[f] (n = 3)	15.0 ± 4.1[f] (n = 4)	2.9 ± 0.9 (n = 3)	6.7 ± 1.9 (n = 3)	0.20	0.45
[CD_3]NNK	3.51	386 ± 304 (n = 3)	59.4 ± 21.5 (n = 3)	<1.4, <0.7, <1.4, 0.21	<0.2, <1.4, <0.9, <2.8	<0.003	<0.02
NNN	2.96	N.Det[g]	N.Det	<1.0, <1.0, 1.9, 4.3	5.9 ± 2.1 (n = 5)		

[a] Male F344 rats were treated 3 times weekly for 4 weeks by s.c. injection and sacrificed 24 h after the last injection. DNA was isolated and analyzed as described in "Materials and Methods."
[b] Mean ± SD.
[c] 0.0167 mmol/kg, except for the higher dose of NNK (0.048 mmol/kg). The dose of 0.0167 mmol/kg is the same as that used in the carcinogenicity assays having a total dose of 1.0 mmol/kg.
[d] <0.4 indicates that HPB was not detected in that sample (less than 2 times background); the detection limit in that sample, based on the amount of DNA analyzed, was 0.4 pmol/μmol guanine. DNA samples ranged from 25–300 μg.
[e] Significantly less than NNK (3.46 mg/kg) olfactory ($P < 0.025$).
[f] Significantly less than NNK (3.46 mg/kg) olfactory ($P < 0.010$) and respiratory ($P < 0.025$). Significantly less than [CD_3]NNK (3.51 mg/kg) olfactory and respiratory ($P < 0.05$).
[g] N.Det, not determined. Previously, we have shown that NNN does not methylate DNA *in vivo* (19).

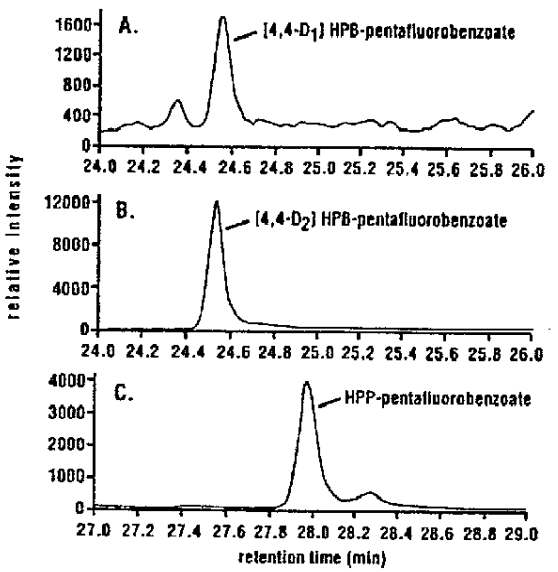

Fig. 3. Detection of HPB-releasing adducts in rats treated with [4,4-D_2]NNK. GC-MS-selected ion monitoring traces of deuterated HPB, as its pentafluorobenzoate derivative, released by acid hydrolysis of DNA isolated from the respiratory portion of the nasal mucosa of a rat treated with [4,4-D_2]NNK. *A*, [4,4-D_1]HPB-pentafluorobenzoate, m/z 360, eluting at 24.56 min; *B*, [4,4-D_2]HPB-pentafluorobenzoate, m/z 361, eluting at 24.53 min; and *C*, HPP-pentafluorobenzoate, m/z 373 (internal standard), eluting at 27.96 min.

in the rats treated with [4,4-D_2]NNK than in the rats treated with equivalent doses of NNK or [CD_3]NNK. No significant difference in HPB-releasing DNA adducts was seen between the olfactory and respiratory mucosa DNA of these rats. HPB-releasing adducts were also detected in the rats treated with the higher dose of NNK and in the respiratory mucosa of the rats treated with NNN.

The GC-MS technique allowed us to assess the extent of exchange of the protons at the 4 position of [4,4-D_2]NNK in the formation of HPB-releasing adducts in DNA. This was accomplished by monitoring m/z 361, corresponding to [4,4-D_2]HPB-pentafluorobenzoate, as well as m/z 360 and 359, in which one or two deuteriums would have exchanged. In five of the six samples of respiratory and olfactory mucosa DNA, levels of HPB-releasing adducts were high enough to permit this analysis. The amount of [4,4-D_1]HPB (m/z 360) detected was 10.5 ± 3.9% of the level of [4,4-D_2]HPB. [4,4-D_0]HPB was detected in only one sample.

The morphological expression of NNK toxicity was evaluated in the rats treated with NNK and its deuterated analogues. Previous studies indicated that toxic morphological degenerative changes may be precursors to nasal tumors (9). In our study, minor histological changes were visible in most rats, including trioctanoin-treated controls (Table 3). These changes may or may not be due to treatment. Toxic lesions of greater intensity were obvious only in the rats treated with the higher dose of NNK. The periodic acid Schiff stain was particularly useful for studying the olfactory basal cells.

DISCUSSION

The comparative carcinogenicity study of NNK, [4,4-D_2]NNK, and [CD_3]NNK carried out previously demonstrated higher carcinogenicity of [4,4-D_2]NNK than the other compounds to the rat nasal mucosa (6). Based on these results, we developed the hypothesis that the higher carcinogenicity of [4,4-D_2]NNK than NNK was due to a shift in metabolism from α-methylene hydroxylation to α-methyl hydroxylation, leading to concomitant lower levels of DNA methylation and

Table 3 *Morphological aspects of toxicity in the nasal mucosa of rats treated with NNK and its deuterated analogues*

Group	Single dose (mg/kg)	No. of rats with nasal mucosa changes[a,b] NSL	+	++	+++
NNK	3.46	1	3	0	0
NNK	9.94	0	0	1	3
[4,4-D_2]NNK	3.49	1	0	2	1
[CD_3]NNK	3.51	2	1	1	0
Trioctanoin control		1	1	2	0

[a] Male F344 rats were treated 3 times weekly for 4 weeks by s.c. injection and sacrificed 24 h later. Histology was carried out as described in "Materials and Methods."
[b] NSL, no significant lesions. +, areas of mild atrophy, vacuolization and desquamation of the olfactory mucosa; ++, as above but more extensive. In addition, involvement of the respiratory mucosa and proliferation of stem cells in the respiratory and olfactory portions; +++, as above but markedly increased with patches of squamous metaplasia and mucoid degeneration in lamina propria.

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higher levels of DNA pyridyloxobutylation than in NNK (6). As a consequence, this would indicate a significant role for DNA pyridyloxobutylation in rat nasal carcinogenesis by NNK. The results of this study support this hypothesis. Under a dosing regimen the same as used in the carcinogenicity study, levels of O^6-mG formed from [4,4-D_2]NNK were significantly lower than those produced from the less carcinogenic compounds, NNK and [CD_3]NNK. In parallel, levels of DNA pyridyloxobutylation, as measured by HPB-releasing adducts, were higher in the rats treated with [4,4-D_2]NNK than in those treated with equivalent doses of NNK or [CD_3]NNK. In the rats treated with NNK, DNA pyridyloxobutylation was clearly detected only at the higher dose; the data indicate that the levels at the lower dose were near the detection limit. The toxicity studies indicated that there was no difference in nasal mucosa toxicity among the rats treated with equimolar doses of NNK, [CD_3]NNK, and [4,4-D_2]NNK, suggesting that toxicity played no role in the higher carcinogenicity of [4,4-D_2]NNK at this dose. Collectively, the results of this study support the hypothesis that DNA pyridyloxobutylation is important in nasal carcinogenesis by NNK.

In a previous study, we also showed that levels of O^6-mG in A/J mouse lung were significantly lower in mice treated with [4,4-D_2]-NNK than in mice treated with either NNK or [CD_3]NNK (30). DNA pyridyloxobutylation was not assessed in that study. The results of this study are consistent with the previous one in demonstrating that substitution of deuterium at the α-methylene carbon of NNK decreases the extent of O^6-mG formation due to the deuterium isotope effect. Effects of deuterium substitution on nitrosamine metabolism have been noted previously (31–36). A difference however between the A/J mouse study and the rat experiments was seen in the relative carcinogenicity of [4,4-D_2]NNK. In A/J mouse lung, it was significantly less tumorigenic than either NNK or [CD_3]NNK, whereas in the rat nasal mucosa it was more carcinogenic (6, 30). This probably results from the important role of DNA methylation in A/J mouse lung tumorigenesis by NNK, as noted in our previous studies as well as those of Belinsky *et al.* (11, 12). In contrast, DNA methylation does not appear to be the critical pathway in rat nasal tumorigenesis by NNK.

The likely role of DNA pyridyloxobutylation in rat nasal carcinogenesis is also supported by the data obtained in the experiments with NNN. As illustrated in Table 1, NNN and NNK have very similar carcinogenic potencies toward the rat nasal mucosa. Both induce primarily benign tumors at the lower total dose of 1.0 mmol/kg, whereas both benign and malignant tumors are observed at the higher total dose of 3.0 mmol/kg. The structure of NNN is such that it is unlikely to methylate DNA, and in previous experiments we have not detected DNA methylation by NNN (19). However, the results of this experiment show for the first time that NNN causes HPB-releasing DNA adducts *in vivo* in the rat nasal mucosa. The levels in the olfactory mucosa were near the detection limit at the dose examined, whereas higher levels were found in the respiratory mucosa. Previous studies have shown that NNN pyridyloxobutylates rat hepatic and A/J mouse lung DNA *in vivo* as well as rat esophageal and nasal mucosa DNA *in vitro* (13, 23, 37, 38).

A somewhat unexpected finding was the significantly lower level of O^6-mG in the olfactory mucosa of the rats treated with the higher dose (9.94 mg/kg) of NNK compared to those treated with the lower dose (3.46 mg/kg). Belinsky *et al.* (7) found an increase in olfactory mucosa O^6-mG as the dose was increased from 1 to 10 mg/kg, using essentially the same dosing regimen as used here, followed by a decrease between 10 and 50 mg/kg. They attributed the decrease to toxicity in the olfactory mucosa at the 50 mg/kg dose. We observed toxicity at the 9.94 mg/kg dose as did Belinsky *et al.* (7). It is unclear why the levels of O^6-mG in the olfactory mucosa are different in the two studies.

Another difference between our results and those obtained previously was the levels of O^6-mG in the olfactory *versus* the respiratory portions of the nasal mucosa in the rats treated with 3.46 mg/kg NNK. We found higher levels of O^6-mG in the olfactory than in the respiratory mucosa, whereas Belinsky *et al.* (7, 9) found the opposite. The protocols used appear to be the same except that in our study the interval between the last treatment and sacrifice was 24 h, whereas in their study it was 4 h. Based on previous results from both our laboratories, the difference in interval until sacrifice would not appear to be important since O^6-mG levels remain relatively stable for up to 24 h following single or multiple doses (5, 29). Presently, we have no explanation for the difference between our results and those of Belinsky *et al.* (7, 9). However, Hong *et al.* (18) did find that microsomes prepared from the olfactory portion of the rat nasal mucosa were more active in catalyzing α-methylene hydroxylation leading to 4-oxo-1-(3-pyridyl)-1-butanone and the DNA methylating species than were microsomes from the respiratory portion. Their results would appear to be consistent with our methylation data.

It is interesting to note that the ratios of HPB-releasing DNA adducts to O^6-mG were higher in the rats treated with the higher dose of NNK and in the rats treated with [4,4-D_2]NNK than in the other groups, as illustrated in Table 2. The higher dose of NNK, totaling 3.0 mmol/kg in the carcinogenicity study, and the dose of [4,4-D_2]NNK totaling 1.0 mmol/kg, produced similar yields of malignant and benign tumors, as noted in Table 1. Thus, higher HPB/O^6-mG ratios were associated with higher yields of malignant tumors. These results are consistent with the hypothesis that DNA pyridyloxobutylation is important in rat nasal carcinogenesis by NNK.

The HPB-releasing DNA adducts measured in this study have been shown to account for at least 50% of the radioactivity in DNA of animals treated with [5-^{3}H]NNK or with NNKOAc *in vitro* (38, 39). The structures of these adducts are not known. Because of their quantitative significance, HPB-releasing adducts are probably a reasonable measure of DNA pyridyloxobutylation, although it is possible that other minor adducts may vary in concentration in ways that are not proportional to the HPB-releasing adducts. Several lines of evidence demonstrate that HPB-releasing adducts or related products of DNA pyridyloxobutylation are biologically significant with respect to mutagenesis and carcinogenesis (40). Two model compounds which pyridyloxobutylate DNA (NNKOAc and CNPB) are potent mutagens in *Salmonella typhimurium* strains, with activities greater than those of the corresponding methylating model compounds acetoxymethylmethylnitrosamine and carbethoxynitrosaminomethane (20, 21). Mutagenicity and DNA adduct studies in bacterial and mammalian cells have indicated that pyridyloxobutylated DNA has intrinsic mutagenic activity as great as that of O^6-mG (21). Recently, we have demonstrated that NNKOAc induces tumors in A/J mouse lung and that these tumors have activated Ki-*ras* oncogenes with G to T transversions and G to A transitions in codon 12 (41). Furthermore, although apparently unstable at the nucleoside level, some HPB-releasing DNA adducts are relatively long lived *in vitro* in double-stranded DNA and *in vivo* in the liver and lung of rats treated with NNK, persisting for at least 4 weeks (22). Collectively, these data provide strong evidence that DNA pyridyloxobutylation is both mutagenic and carcinogenic, supporting the hypothesis presented here that DNA pyridyloxobutylation is a key step in rat nasal carcinogenesis by tobacco-specific nitrosamines. An additional mechanism by which DNA pyridyloxobutylation could contribute to rat nasal carcinogenesis by NNK involves its ability to inhibit the repair of O^6-mG, as recently demonstrated by

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Peterson *et al.* (42). Nasal tumors are induced in rats by NNK using a protocol of three times weekly s.c. injections for 20 weeks, followed by no further treatment until tumors appear. It is possible that after cessation of NNK treatment, pyridyloxobutylated DNA inhibits O^6-mG repair through inhibition of the repair enzyme O^6-alkyl-guanine-DNA-alkyltransferase. Data supporting this concept have been presented previously by Belinsky *et al.* (29).

Previous studies of the solvolysis of CNPB and NNKOAc demonstrated general base catalyzed exchange of the protons on the methylene carbon adjacent to the nitroso group, indicating that 4-(3-pyridyl)-4-oxobutanediazohydroxide is in equilibrium with the corresponding diazonium ion and diazo compound (43). Similar results have been obtained in solvolysis experiments with acetoxymethylmethylnitrosamine and carbethoxynitrosaminomethane (44). The detection of [4,4-D_1]HPB released from nasal mucosa DNA of rats treated with [4,4-D_2]NNK indicates that these equilibria occur *in vivo*. Because of the deuterium isotope effect, the extent of exchange in rats treated with NNK is probably greater than the value of 10.5% observed for [4,4-D_2]NNK. Deuterium exchange was not observed upon mass spectrometric analysis of 7-methylguanine and 7-ethylguanine isolated from DNA of rats treated with deuterated *N*-nitrosodimethylamine or *N*-nitrosodiethylamine (45, 46).

In conclusion, the results of this study are consistent with the hypothesis that DNA pyridyloxobutylation is important in rat nasal mucosa carcinogenesis by tobacco-specific nitrosamines.

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